



RECEIPT  
#8

Dkt. 43016-A-PCT-US/JPW/SHS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Philip O. Livingston and Friedhelm Helling  
U.S. Serial No.: 08/196,154 Group Unit: 1806  
Filed : November 16, 1995 Examiner: A. Caputa  
For : GANGLIOSIDE-KLH CONJUGATE VACCINES WITH QS-21

1185 Avenue of the Americas  
New York, New York 10036  
June 23, 1999

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

COMMUNICATION REQUESTING CORRECTED FILING RECEIPT

This Communication is filed to request a corrected Filing Receipt in connection with the above-identified application. Upon receipt of the official Filing Receipt for the subject application, copy of which is attached hereto as **Exhibit A**, applicant's undersigned attorney noticed errors.

Applicants hereby respectfully request that a corrected Filing Receipt be issued. Specifically, after "APPLICANT(S)", the following now appears:

PHILLIP O. LIVINGSTON, NEW YORK, NY; FRIEDHELM HELLING,  
NEW YORK, NY

A corrected Filing Receipt should read as follows:

--PHILIP O. LIVINGSTON, NEW YORK, NY;  
FRIEDHELM HELLING, NEW YORK, NY--

RECEIVED  
14 1999  
MAIL ROOM

Further, after "CONTINUING DATA AS CLAIMED BY APPLICANT-", the following now appears:

Applicants : Philip Livingston and Friedhelm Helling  
Serial No. : 08/196,154  
Filed : June 7, 1995  
Page 2

THIS APPLN IS A 371 OF PCT/US94/00757 01/21/94  
AND A CON OF 08/009,628 01/21/93 PAT 5,333,920

A corrected Filing Receipt should read as follows:

--THIS APPLN IS A 371 OF PCT/US94/00757 01/21/94  
AND A CIP OF 08/009,268 01/22/93 ABN--

Also, after "TITLE", the following now appears:

GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS OS-21

A corrected Filing Receipt should read as follows:

--GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS QS-21--

Applicants contend that the changes to the Filing Receipt are due to clerical errors made by the PTO and that the correct inventor's name, continuation information and title of the application may be found in the Declaration and Power of Attorney filed November 14, 1995. A copy of the Declaration and Power of Attorney is attached hereto as **Exhibit B**. Accordingly, applicants request that a corrected Filing Receipt be issued.

Furthermore, applicants attach hereto as **Exhibit C** a copy of the filing receipt in connection with related application 08/477,097, filed June 7, 1995 (identified by our Docket 43016-B). Applicants contend that this is prima facie evidence of the fact that the continuing data on the filing receipt should read that it claim priority of U.S. 08/009,268, filed January 22, 1993.

Furthermore, applicants attach hereto as **Exhibit D** a copy of Communication Inquiring as to Copendency of Application which is was filed in connection with U.S. Serial No 08/009,268, filed January 22, 1993, which is the parent application to the subject

Applicants : Philip Livingston and Friedhelm Helling  
Serial No. : 08/196,154  
Filed : June 7, 1995  
Page 3

application and identified by our Docket No. 43016. This Communication requests the revival of U.S. Serial No 08/009,268 if it is deemed necessary.

No fee is deemed necessary in connection with the filing of this Communication. However, if any fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

*Albert Wai Kit Chan*

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

*Albert Wai Kit Chan* 6/23/99  
Albert Wai-Kit Chan Date  
Reg. No. 36,479

John P. White  
Registration No. 28,678  
Albert Wai-Kit Chan  
Registration No. 36,479  
Attorneys for Applicant(s)  
Cooper & Dunham, LLP  
1185 Avenue of the Americas  
New York, New York 10036  
(212) 278-0400

FILING RECEIPT



11/ARC/MSK  
UNITED STATES DEPARTMENT OF COMMERCE  
Patent and Trademark Office  
ASSISTANT SECRETARY AND COMMISSIONER  
OF PATENTS AND TRADEMARKS  
Washington, D.C. 20231

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTORNEY DOCKET NO.	DRWGS	TOT CL	IND CL
08/196,154	11/16/95	1806	\$726.00	43016-A-PCT-	26	23	0

JOHN P WHITE  
COOPER AND DUNHAM  
1185 AVENUE OF THE AMERICAS  
NEW YORK NY 10036

Receipt is acknowledged of this nonprovisional Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please write to the Application Processing Division's Customer Correction Branch within 10 days of receipt. Please provide a copy of the Filing Receipt with the changes noted thereon.

Applicant(s)

PHILLIP O. LIVINGSTON, NEW YORK, NY; FRIEDHELM HELLING,  
NEW YORK, NY.

CONTINUING DATA AS CLAIMED BY APPLICANT-

THIS APPLN IS A 371 OF PCT/US94/00757 01/21/94  
AND A CON OF 08/009,628 01/21/93 PAT 5,333,920

FOREIGN FILING LICENSE GRANTED 03/30/96

\* SMALL ENTITY \*

TITLE

GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS OS-21

PRELIMINARY CLASS: 424

SEP 13 1996

DOCKET CLERK

## Declaration and Power of Attorney

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

GANGLIOSIDE-KLH CONJUGATE VACCINES WITH QS-21

the specification of which  
(check one)

\_\_\_\_\_ is attached hereto.

x was filed on January 21, 1994 23

Application Serial No. PCT/US94/00757 and entered the National  
stage as U.S. Serial No. 08/196,154  
and was amended on July 21, 1995  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information of which I am aware which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)		Filing Date	Priority Claimed	
Number	Country		Yes	No
None	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Sections 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u>Application Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
<u>PCT/US94/00757</u>	<u>January 21, 1994</u>	<u>pending</u>
<u>08/099,268</u>	<u>January 22, 1993</u>	<u>abandoned</u>

And I hereby appoint

John P. White (Reg. No. 28,678); Thomas F. Moran (Reg. No. 16,579); Norman H. Zivin (Reg. No. 25,385); Ivan S. Kavrukov (Reg. No. 25,161); Christopher C. Dunham (Reg. No. 22,031); Thomas G. Carulli (Reg. No. 30,616); Robert D. Katz (Reg. No. 30,141); Peter J. Phillips (Reg. No. 29,691); Richard S. Milner (Reg. No. 33,970); Matthew J. Golden (Reg. No. 35,161); Albert Wai-Kit Chan (Reg. No. 36,479); Kristina L. Konstas (Reg. No. 37,864); Robert T. Maldonado (Reg. No. 38,232); and Lewis J. Kreisler (Reg. No. 38,522),

and each of them, all c/o Cooper & Dunham LLP of 1185 Avenue of the Americas, New York, New York 10036, Tel. (212) 278-0400, my attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International Applications which are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to

John P. White Reg. No. 28,678  
Cooper & Dunham LLP  
1185 Avenue of the Americas  
New York, New York 10036  
Tel (212) 278-0400

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first joint inventor Philip Ordway Livingston  
 Inventor's signature Philip Livingston  
 Citizenship United States Date of signature 8/24/95  
 Residence 156 E 79th Street, New York, NY 10021  
 Post Office Address same as residence address

Full name of joint  
inventor (if any) Friedhelm Helling  
Inventor's signature Friedhelm Helling  
Citizenship Germany Date of signature 8/17/95  
Residence 504 E 63rd Street, Apt 25N, New York, NY 10021  
Post Office Address same as residence address

Full name of joint  
inventor (if any) \_\_\_\_\_  
Inventor's signature \_\_\_\_\_  
Citizenship \_\_\_\_\_ Date of signature \_\_\_\_\_  
Residence \_\_\_\_\_  
Post Office Address \_\_\_\_\_

Full name of joint  
inventor (if any) \_\_\_\_\_  
Inventor's signature \_\_\_\_\_  
Citizenship \_\_\_\_\_ Date of signature \_\_\_\_\_  
Residence \_\_\_\_\_  
Post Office Address \_\_\_\_\_

FILING RECEIPT  
CORRECTED



*JPL 42/103K*  
UNITED STATES DEPARTMENT OF COMMERCE  
Patent and Trademark Office  
ASSISTANT SECRETARY AND COMMISSIONER  
OF PATENTS AND TRADEMARKS  
Washington, D.C. 20231

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTORNEY DOCKET NO.	DRWGS	TOT CL	IND CL
08/477,097	06/07/95	1806	\$904.00	43016-B/JPW/	26	22	1

JOHN P WHITE  
COOPER & DUNHAM  
1185 AVENUE OF THE AMERICAS  
NEW YORK NY 10036

Receipt is acknowledged of this nonprovisional Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please write to the Application Processing Division's Customer Correction Branch within 10 days of receipt. Please provide a copy of the Filing Receipt with the changes noted thereon.

**Applicant(s)**

PHILIP O. LIVINGSTON, NEW YORK, NY; FRIEDHELM HELLING,  
NEW YORK, NY.

CONTINUING DATA AS CLAIMED BY APPLICANT-

THIS APPLN IS A CON OF PCT/US94/00757 01/21/94  
WHICH IS A CON OF 08/009,268 01/22/93 ABN

FOREIGN FILING LICENSE GRANTED 09/20/95  
TITLE  
GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS QS-21

PRELIMINARY CLASS: 424

JUL - 8 1995



**THE BOARD OF PATENT APPEALS AND INTERFERENCES**

Applicants : Philip O. Livingston and Friedhelm Helling  
U.S. Serial No.: 08/009,268 Group Unit: 1813  
Filed : January 22, 1993 Examiner: Kresek Staples  
For : GANGLIOSIDE-KLH CONJUGATE VACCINES WITH QS-21

1185 Avenue of the Americas  
New York, New York 10036  
June 23, 1999

Sir:

**COMMUNICATION INQUIRING AS TO COPENDENCY STATUS OF APPLICATION**

This Communication is submitted to ensure that applications which claim the priority the subject application are eligible for withdrawal of finality under 37 C.F.R. 1.129(a). In the event that copendency between the subject application and PCT/US94/00757 is required for these applications to be eligible for withdrawal of finality under 37 C.F.R. 1.129(a), and if it is determined that there was no copendency, applicants hereby petition to revive the subject application under 37 C.F.R. 1.137(b).

**I. Background**

The following applications claim priority of the subject application:

- 1) U.S. Serial No. 08/196,154, filed November 16, 1995 (Our Docket 43016-A-PCT-US);
- 2) U.S. Serial No. 08/477,097, filed June 7, 1995 (Our Docket 43016-B);
- 3) U.S. Serial No. 08/475,784, filed June 7, 1995 Docket 43016-C);
- 4) U.S. Serial No. 08/477,147, filed June 7, 1995 (Our

Applicants : Philip O. Livingston and Friedhelm Helling  
U.S. Serial No.: 08/009,268  
Filed : January 22, 1993  
Page 2

Docket 43016-D); and

- 5) U.S. Serial No. 08/481,809, filed June 7, 1995 (Our Docket 43016-E).

In the event that the subject application was not copending with PCT/US94/00757, applicants hereby submit this petition under 37 C.F.R. § 1.137(b).

**Petition under 37 C.F.R. 1.137(b)**

A petition under 37 C.F.R. 1.137(b) requires: (a) the required reply, unless previously filed; (b) the petition fee as set forth in 37 C.F.R. 1.17(m); (c) a statement that the entire delay in filing the required reply from the due date for the reply until the filing of a grantable petition pursuant to 37 C.F.R. 1.137(b) was unintentional; (d) any terminal disclaimer required pursuant to 37 C.F.R. 1.137(c).

**Required Reply**

In the event that it is determined that the revival of the subject application results in the application being pending as of the last day of the statutory period for filing a response, i.e. November 2, 1993, and if an extension of time of three (3) months is needed to ensure copendency with PCT/US94/00757, applicants hereby petition for a three month extension of time in connection with the subject application. Applicants have previously established small entity status. The current required for a three month extension of time is \$435.00 and authorization is hereby given to charge this amount to Deposit Account No. 03-3125, if it is deemed necessary. Accordingly, the subject application would have been pending at the time PCT/US94/00757 was filed.

Applicants : Philip O. Livingston and Friedhelm Helling  
U.S. Serial No.: 08/009,268  
Filed : January 22, 1993  
Page 3

Petition fee

The fee under 37 C.F.R. 1.17(m) for a petition to revive an unintentionally abandoned application for a small entity is \$605.00 and authorization is hereby given to charge this amount to Deposit Account No. 03-3125, if it is deemed necessary.

Statement of unintentional delay

Applicants contend that entire delay in filing the required reply from the due date for the reply until the filing of a grantable petition pursuant to 37 C.F.R. 1.137 (b) was unintentional.

The United States Patent and Trademark Office issued an office action on August 2, 1993 in connection with the subject application. A response was due November 2, 1993. However, applicants did not respond to the office action. Accordingly, the United States Patent and Trademark Office issued a Notice of Abandonment in connection with the subject application on March 9, 1994.

On January 21, 1994, applicants filed International Application No PCT/US94/00757 (our docket 43106-A-PCT) and claimed priority of the above identified application. Applicants believed that the subject application was still pending at the time that PCT/US94/00757 was filed. In support, applicants attach hereto, as Exhibit 1, a copy of the Request filed in connection with PCT/US94/00757 which states on page 3 that the priority of U.S. Serial No. 08/009,268 was claimed.

Applicants contend that the subject application was unintentionally abandoned. Applicants believed that the subject application was still pending at the time PCT/US94/00757 was filed. If they had realized that their failure to respond to the office action within the statutory period would have resulted in a lack of copendancy

Applicants : Philip O. Livingston and Friedhelm Helling  
U.S. Serial No.: 08/009,268  
Filed : January 22, 1993  
Page 4

between the subject application and PCT/US94/00757, they would have responded. Applicants respectfully point out that a response to the outstanding office action would have been due February 2, 1994 had they petitioned for a three-month extension of time. If the subject application was pending on February 2, 1994, then there would have been copendency with PCT/US94/00757.

Applicants noticed the possible lack of pendency between the subject application and PCT/US94/00757 as follows. Applicants had tried to correct filing receipts in connection with applications which claim priority of the subject application. Applicants were trying to correct the filing receipts to reflect that they claimed priority of the subject application. However, applicants did not receive responses from the United States Patent and Trademark Office regarding the request for corrected filing receipts. Upon further review of the prosecution history of the subject application, applicants realized that the subject application may have been unintentionally abandoned prior to the filing of PCT/US94/00757. Accordingly, if there is a lack of pendency between these applications, applicants hereby petition to revive the subject application as an unintentionally abandoned application. Accordingly, applicants contend that entire delay in filing the required reply from the due date for the reply until the filing of a grantable petition was unintentional.

#### Terminal Disclaimer

37 C.F.R. 1.137(c) requires that a petition under 37 C.F.R. 1.137(b) be accompanied by a terminal disclaimer in a nonprovisional application filed before June 8, 1995. However, applicants respectfully contend that the requirement for a terminal disclaimer is not appropriate under the present circumstances. Applicants respectfully direct the Examiner's attention to MPEP

Applicants : Philip O. Livingston and Friedhelm Helling  
U.S. Serial No.: 08/009,268  
Filed : January 22, 1993  
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711.03(c) (III) (G) which states that:

"in the event that an applicant considers the requirement for a terminal disclaimer to be inappropriate under the circumstances of the application at issue, the applicants should file a petition under 37 C.F.R. 1.183 and the petition fee, to request a waiver of this requirement....The grant of such a petition, however, is strictly limited to situations wherein applicant has made a showing of an "extraordinary situation" in which "justice requires" the requested relief. Such situations are namely when: (A) the abandonment of the application caused no actual delay in prosecution (e.g., application revived solely for copendency with a continuing application whose prosecution was unaffected by the abandonment)...

Applicants respectfully point out that they are petitioning to revive the subject application solely to ensure that there was copendency between the subject application and PCT/US94/00757. Accordingly, applicants contend that the above example presented in the MPEP accords with the facts of the subject application. Applicants contend that the prosecution of PCT/US94/00757 has not been affected by the abandonment.

Applicants also point out that in a April 28, 1999 telephone conference, Ms. Lissi Mojica of the Office of Petitions of the United States Patent and Trademark Office indicated to Mr. Spencer Schneider of the undersigned attorney's office that the terminal disclaimer requirement could be waived under the facts of the subject application.

In support and in conformity with MPEP 711.03(c) (III) (G), applicants attach hereto as Exhibit 2, a Petition under 37 C.F.R. 1.183 and authorization is hereby given to charge the \$130.00 petition fee to Deposit Account No. 03-3125, if it is deemed necessary. Applicants contend that this satisfies the requirements such that the terminal disclaimer requirement should be waived.

Applicants : Philip O. Livingston and Friedhelm Helling  
U.S. Serial No.: 08/009,268  
Filed : January 22, 1993  
Page 6

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee is deemed necessary in connection with the filing of this Communication. However, if any fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

Albert Wai Kit Chan

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.	
<u>Albert Wai Kit Chan</u> Albert Wai-Kit Chan Reg. No. 36,479	<u>6/2/97</u> Date

John P. White  
Registration No. 28,678  
Albert Wai-Kit Chan  
Registration No. 36,479  
Attorney for Applicants  
Cooper & Dunham LLP  
1185 Avenue of the Americas  
New York, New York 10036  
(212) 278-0400

# PCT

## REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference  
(if desired, 12 characters maximum)

43016-A-PCT

### Box No. I TITLE OF INVENTION

GANGLIOSIDE-KLH CONJUGATE VACCINES PLUS OS-21

### Box No. II APPLICANT

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH  
1275 York Avenue  
New York, New York 10021  
United States of America

☐ This person is also inventor

Telephone No.

NONE

Facsimile No.

NONE

Teleprinter No.

NONE

State (i.e. country) of nationality:

United States of America

State (i.e. country) of residence:

United States of America

This person is applicant for the purposes of:

☐

all designated States

☒

all designated States except the United States of America

☐

the United States of America only

☐

the States indicated in the Supplemental Box

### Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

LIVINGSTON, PHILIP O.  
156 East 79th Street  
Apartment 6C  
New York, New York 10021  
United States of America

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only (If this check-box is marked, do not fill in below.)

State (i.e. country) of nationality:

United States of America

State (i.e. country) of residence:

United States of America

This person is applicant for the purposes of:

☐

all designated States

☐

all designated States except the United States of America

☒

the United States of America only

☐

the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

HELLING, FRIEDHELM  
303 East 71st Street  
Apartment 6H  
New York, New York 10021  
United States of America

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only (If this check-box is marked, do not fill in below.)

State (i.e. country) of nationality:

Germany

State (i.e. country) of residence:

United States of America

This person is applicant for the purposes of:

☐

all designated States

☐

all designated States except the United States of America

☒

the United States of America only

☐

the State the Supo

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

**Box No. IV AGENT OR COMMON REPRESENTATIVE: OR ADDRESS FOR CORRESPONDENCE**

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:

☒ agent ☐ common representative

Name and address: *(Family name followed by given name for a legal entity, full official designation. The address must include postal code and name of country.)*

Telephone No  
(212)977-9550

WHITE, JOHN P.  
Cooper & Dunham  
30 Rockefeller Plaza  
New York, New York 10112  
United States of America

Facsimile No  
(212)664-0525

Teletypewriter No  
422523 COOP UI

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

**Box No. V DESIGNATION OF STATES**

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

**Regional Patent**

☒ EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, DE Germany, DK Denmark, ES Spain, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☐ OA **OAPI Patent:** Benin, Burkina Faso, Cameroon, Central African Republic, Chad, Congo, Côte d'Ivoire, Gabon, Guinea, Mali, Mauritania, Senegal, Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line)

**National Patent** (if other kind of protection or treatment desired, specify on dotted line):

<input type="checkbox"/> AT Austria	<input type="checkbox"/> MG Madagascar
<input checked="" type="checkbox"/> AU Australia	<input type="checkbox"/> MN Mongolia
<input type="checkbox"/> BB Barbados	<input type="checkbox"/> MW Malawi
<input type="checkbox"/> BG Bulgaria	<input type="checkbox"/> NL Netherlands
<input type="checkbox"/> BR Brazil	<input checked="" type="checkbox"/> NO Norway
<input checked="" type="checkbox"/> CA Canada	<input checked="" type="checkbox"/> NZ New Zealand
<input type="checkbox"/> CH and LI Switzerland and Liechtenstein	<input type="checkbox"/> PL Poland
<input type="checkbox"/> CZ Czech Republic	<input type="checkbox"/> PT Portugal
<input type="checkbox"/> DE Germany	<input type="checkbox"/> RO Romania
<input type="checkbox"/> DK Denmark	<input checked="" type="checkbox"/> RU Russian Federation
<input type="checkbox"/> ES Spain	<input type="checkbox"/> SD Sudan
<input checked="" type="checkbox"/> FI Finland	<input type="checkbox"/> SE Sweden
<input type="checkbox"/> GB United Kingdom	<input type="checkbox"/> SK Slovak Republic
<input checked="" type="checkbox"/> HU Hungary	<input type="checkbox"/> UA Ukraine
<input checked="" type="checkbox"/> JP Japan	<input checked="" type="checkbox"/> US United States of America
<input type="checkbox"/> KP Democratic People's Republic of Korea	(continuation-in-part)
<input checked="" type="checkbox"/> KR Republic of Korea	Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:
<input type="checkbox"/> LK Sri Lanka	<input type="checkbox"/>
<input type="checkbox"/> LU Luxembourg	<input type="checkbox"/>

In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of \_\_\_\_\_  
The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.



<b>Box No. VI PRIORITY CLAIM</b>		Further priority claims are indicated in the Supplemental Box <input type="checkbox"/>	
The priority of the following earlier application(s) is hereby claimed:			
Country <small>(in which, or for which, the application was filed)</small>	Filing Date <small>(day/month/year)</small>	Application No.	Office of filing <small>(only the regional or international office)</small>
Item (1) United States of America	22 January 1993	08/009,268	
Item (2)			
Item (3)			
<p>Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):</p> <p><input checked="" type="checkbox"/> The receiving Office is hereby requested to prepare and transmit to the International Serial Number 08/009,268 Bureau a certified copy of the earlier application(s) identified above as item(s)</p>			
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<b>Box No. VIII CHECK LIST</b>			
<p>This international application contains the following number of sheets:</p> <p>1. request : 4 sheets</p> <p>2. description : 143 sheets</p> <p>3. claims : 5 sheets</p> <p>4. abstract : 1 sheet</p> <p>5. drawings : 26 sheets</p> <p style="text-align: right;">Total : 179 sheets</p>		<p>This international application is accompanied by the item(s) marked below:</p> <p>1. <input type="checkbox"/> separate signed power of attorney      5. <input checked="" type="checkbox"/> fee calculation sheet</p> <p>2. <input type="checkbox"/> copy of general power of attorney      6. <input type="checkbox"/> separate indications concerning deposited microorganisms</p> <p>3. <input type="checkbox"/> statement explaining lack of signature      7. <input type="checkbox"/> nucleotide and/or amino acid sequence listing (diskette)</p> <p>4. <input type="checkbox"/> priority document(s) identified in Box No. VI as item(s):      8. <input checked="" type="checkbox"/> other (specify):</p> <p style="text-align: right;">Assignment</p>	
Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.			
<b>Box No. IX SIGNATURE OF APPLICANT OR AGENT</b>			
<p>Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).</p> <p style="text-align: center;">SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH</p> <p>BY: <u>James S. Quirk</u>      DATE: <u>1/21/94</u></p> <p>NAME: Mr. James S. Quirk</p> <p>TITLE: Senior Vice President</p>			

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## Box No. VI PRIORITY CLAIM

Further priority claims are: ☐ filed in the Supplemental Box

The priority of the following earlier application(s) is hereby claimed:

Country (in which, or for which, the application was filed)	Filing Date (day/month/year)	Application No.	Office of filing (only for regional or international application)
item (1) United States of America	22 January 1993	08/009,268	
item (2)			
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

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Country (or regional Office): United States of America Date (day/month/year): 22 January 1993 Number: 08/009,268

## Box No. VIII CHECK LIST

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1. request : 4 sheets  
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Total : 179 sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney  
 2. ☐ copy of general power of attorney  
 3. ☐ statement explaining lack of signature  
 4. ☐ priority document(s) identified in Box No. VI as item(s):  
 5. ☒ fee calculation sheet  
 6. ☐ separate indications concerning deposited microorganisms  
 7. ☐ nucleotide and/or amino acid sequence listing (diskette)  
 8. ☒ other (specify): Assignment

Figure No. \_\_\_\_\_ of the drawings (if any) should accompany the abstract when it is published.

## Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request):

Philip Livingston MD 1/21/94  
 Philip Livingston Date

Friedhelm Helling 1/21/94  
 Friedhelm Helling Date

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**THE BOARD OF PATENT APPEALS AND INTERFERENCES**

Applicants : Philip O. Livingston and Friedhelm Helling  
U.S. Serial No.: 08/009,268 Group Unit: 1813  
Filed : November 16, 1995 Examiner: Kresek Staples  
For : GANGLIOSIDE-KLH CONJUGATE VACCINES WITH QS-21

1185 Avenue of the Americas  
New York, New York 10036  
June 23, 1999

Sir:

**PETITION UNDER 37 C.F.R. § 1.183 TO SUSPEND THE RULES**

This Petition under 37 C.F.R. 1.183 is submitted pursuant to MPEP 711(c)(III)(G) which states that in the event that applicant considers the requirement for a terminal disclaimer to be inappropriate under the circumstances of the application at issue, the applicant should file a petition under 37 C.F.R. 1.183 (and petition fee) to request a waiver of this requirement. Authorization is hereby given to charge the \$130.00 fee to Deposit Account No. 03-3125 enclose the \$130.00 fee under 37 C.F.R. 1.17(h) for petitions to the commissioner, unless otherwise specified.

MPEP 711(c)(III)(G) states:

The grant of such a petition, however, is strictly limited to situations wherein applicant has made a showing of an "extraordinary situation" in which "justice requires" the requested relief. Such situations are namely when: (A) the abandonment of the application caused no actual delay in prosecution (e.g., application revived solely for copendancy with a continuing application whose prosecution was unaffected by the abandonment)...

Applicants contend that the above example presented in the MPEP accords with the facts of the subject application. Applicants point out that they are reviving the subject application solely to

Applicants : Philip O. Livingston and Friedhelm Helling  
U.S. Serial No.: 08/009,268  
Filed : November 16, 1995  
Page 2

maintain its pendency with PCT/US94/00757. Applicants contend that the prosecution of PCT/US94/00757 has not been affected by the abandonment. Applicants also point out that in a April 28, 1999 telephone conference, Ms. Lissi Mojica of the Office of Petitions of the United States Patent and Trademark Office indicated to Mr. Spencer Schneider of the undersigned attorney's office that the terminal disclaimer requirement could be waived under the facts of the subject application.

Accordingly, applicants hereby petition that the rules be suspended such that the terminal disclaimer requirement be waived.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee is deemed necessary. However, if any fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

Albert Wai-Kit Chan

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.	
<u>Albert Wai-Kit Chan</u>	<u>6/28/99</u>
Albert Wai-Kit Chan Reg. No. 36,479	Date

John P. White  
Registration No. 28,678  
Albert Wai-Kit Chan  
Registration No. 36,479  
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Cooper & Dunham LLP  
1185 Avenue of the Americas  
New York, New York 10036  
(212) 278-0400



US005102663A

**United States Patent** [19]

Livingston et al.

[11] **Patent Number:** **5,102,663**[45] **Date of Patent:** **Apr. 7, 1992****[54] VACCINE FOR STIMULATING OR ENHANCING PRODUCTION OF ANTIBODIES AGAINST 9-O-ACETYL GD3****[75] Inventors:** Philip O. Livingston; Gerd J. Ritter, both of New York, N.Y.; Herbert F. Oettgen, New Canaan, Conn.; Lloyd J. Old, New York, N.Y.**[73] Assignee:** Sloan-Kettering Institute for Cancer Research, New York, N.Y.**[21] Appl. No.:** 259,182**[22] Filed:** Oct. 18, 1988**[51] Int. Cl.<sup>5</sup>** ..... A61K 39/39; A61K 37/20; A61K 37/22; A61K 39/395**[52] U.S. Cl.** ..... 424/88; 424/85.8; 424/422; 424/423; 514/25; 514/885; 530/806; 530/842; 530/387.5; 530/389.7; 436/23; 436/503; 436/813; 436/822; 436/823**[58] Field of Search** ..... 424/88, 85.8, 422, 423; 514/25, 885; 530/387, 806, 842; 436/23, 503, 813, 822, 823**[56] References Cited****U.S. PATENT DOCUMENTS**4,849,509 7/1989 Thurin et al. .... 530/387.  
4,918,164 4/1990 Hellstrom et al. .... 530/387**OTHER PUBLICATIONS**Cheresh, D. A. et al., *J. Biol. Chem.* 12:7453-7459, 1984.Pinsky, C. M. et al., *Rec. Results Cancer Res.* 47:37-41, 1974.Haverkamp, J. et al., *Hoppe-Seyler's Z. Physiol. Chem.* 356:1575-1583.Momoi, T. et al., *Hoppe-Seyler's Z. Physiol. Chem.* 361:1201-1210.H. Towbin et al., (1984) *Journal of Immunological Methods*, 72:471-479.R. W. Ledeen et al., (1973) *Journal of Neurochemistry*, 21:829-839.Tadashi Tai, (1985) *Int. J. Cancer*, 35:607-612.Leslie D. Cahan et al., (1982) *Proc. Natl. Acad. Sci.*, 79:7629-7633.Roland Schauer (1978) *Characterization of Sialic Acids*.In. V. Ginsburg (ed.), *Methods of Enzymology*, vol. L., part C:64-89.Hiroshi Yamaguchi et al., (1987) *Proc. Natl. Acad. Sci.*, 84:2416-2420.Makoto Ito and Tatsuya Yamagata (1986) *Journal of Biological Chemistry*, 261(30): 14278.Philip O. Livingston et al., (1987) *The Journal of Immunology*, 138(5): 1524.Nai-Kong V. Cheung et al., (1986) *Journal of Clinical Oncology*, 5(9):1430.Philip O. Livingston et al., (1986) *Proc. Natl. Acad. Sci. U.S.A.* 84:2911.Alan N. Houghton et al., (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82:1242.Reiko F. Irie and Donald L. Morton (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83:8694.Andrew S. Blum et al., (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:8716-8720.Tetsuya Tsuchida et al., (1987) *JNCI* 78(1): 45-50.Tetsuya Tsuchida et al., (1987) *JNCI* 78(1) 55-60.Wolfgang G. Dippold et al., (1980) *Proc. Natl. Acad. Sci. U.S.A.* 70(10): 6114-6118.Robert K. Yu et al., (1985) *J. Biochem.* 98:1367-1373.Su-Chen Li et al., (1975) *Journal of Biological Chemistry*, 250(17): 6786-6791, 1975.

(List continued on next page.)

*Primary Examiner*—Thurman K. Page*Assistant Examiner*—Carlos Azpuru*Attorney, Agent, or Firm*—John P. White**[57]****ABSTRACT**

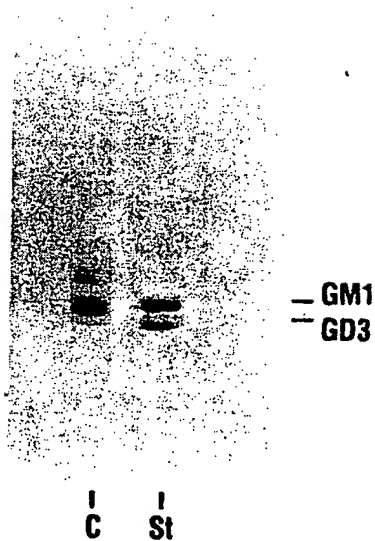
The present invention provides a vaccine for stimulating or enhancing in a subject to whom the vaccine is administered, production of antibodies directed against 9-O-acetyl GD3 ganglioside comprising an amount of purified 9-O-acetyl GD3 ganglioside effective to stimulate or enhance antibody production in the subject and a pharmaceutically acceptable carrier. This invention also provides purified ganglioside and ganglioside mixtures which comprise 9-O-acetyl GD3 ganglioside and one or more additional acetyl groups.

**22 Claims, 9 Drawing Sheets**

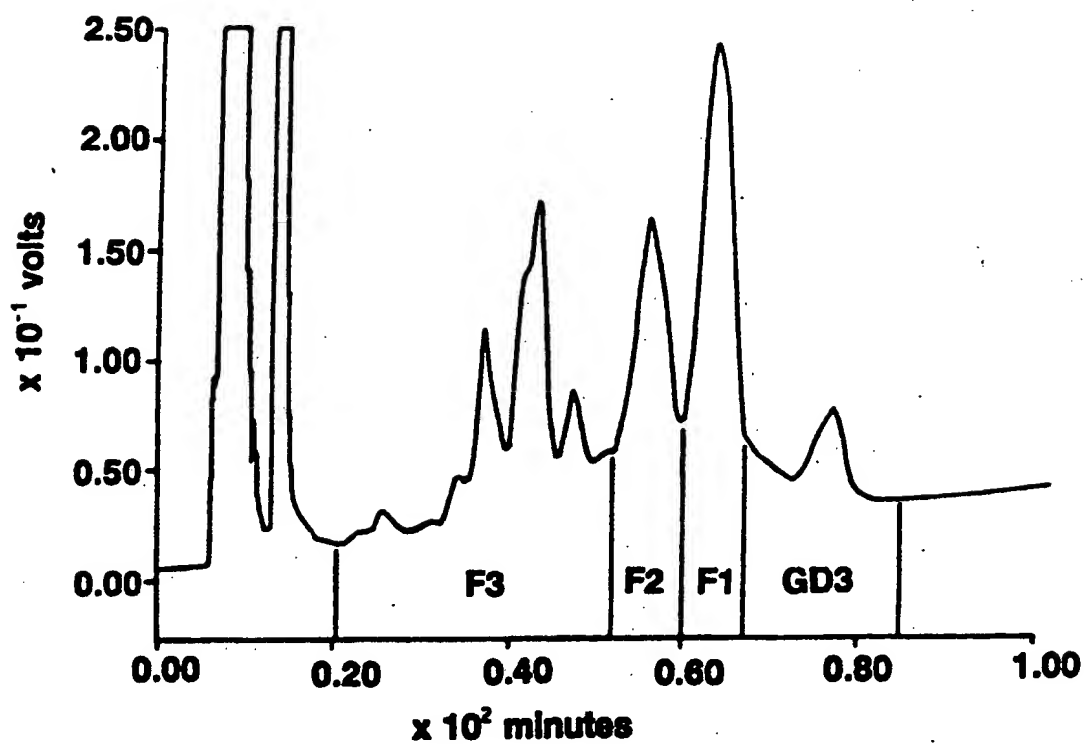
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FIGURE 1



**O-acetylation of GD3  
monitored by HPTLC**

**FIGURE 2**

**Preparative separation of O-acetyl-GD3 derivatives by HPLC  
( $\text{NH}_2\text{-col}$ ; acetonitrile/phosphate buffer gradient)**



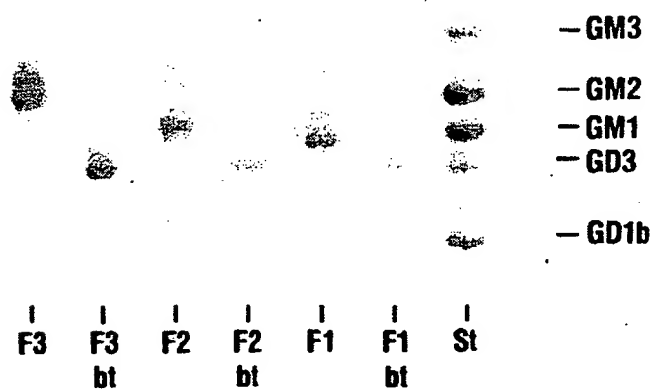
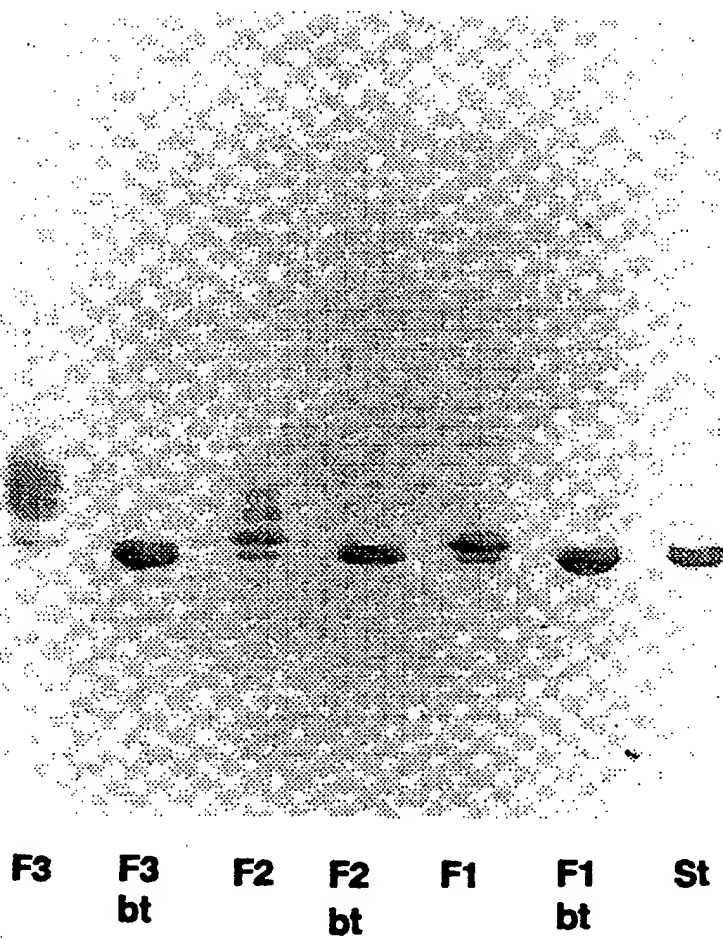
**FIGURE 3****HPTLC of isolated O-acetyl-GD3 derivatives**

FIGURE 4

R24



ITLC of 0-acetyl-GD3 derivatives

FIGURE 5

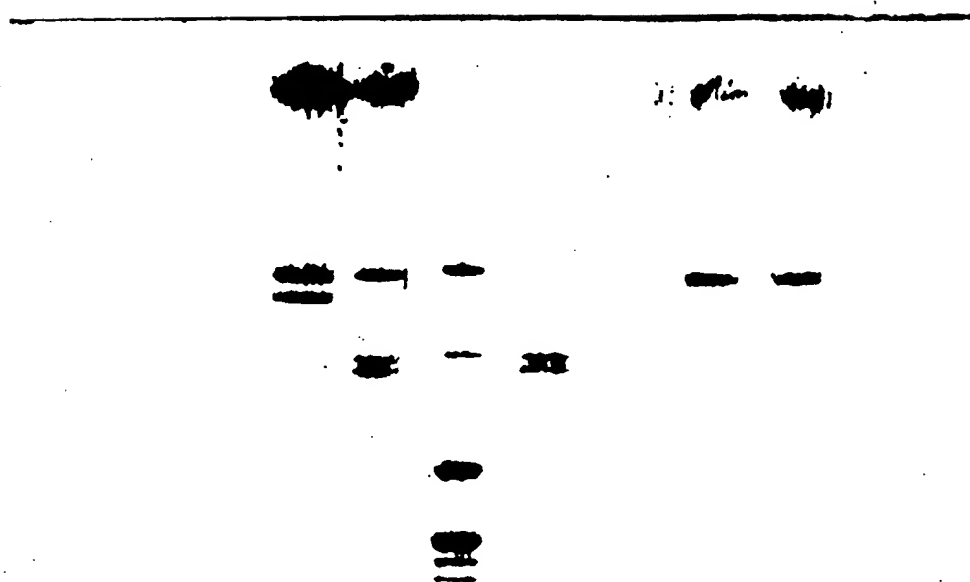


FIGURE 6A

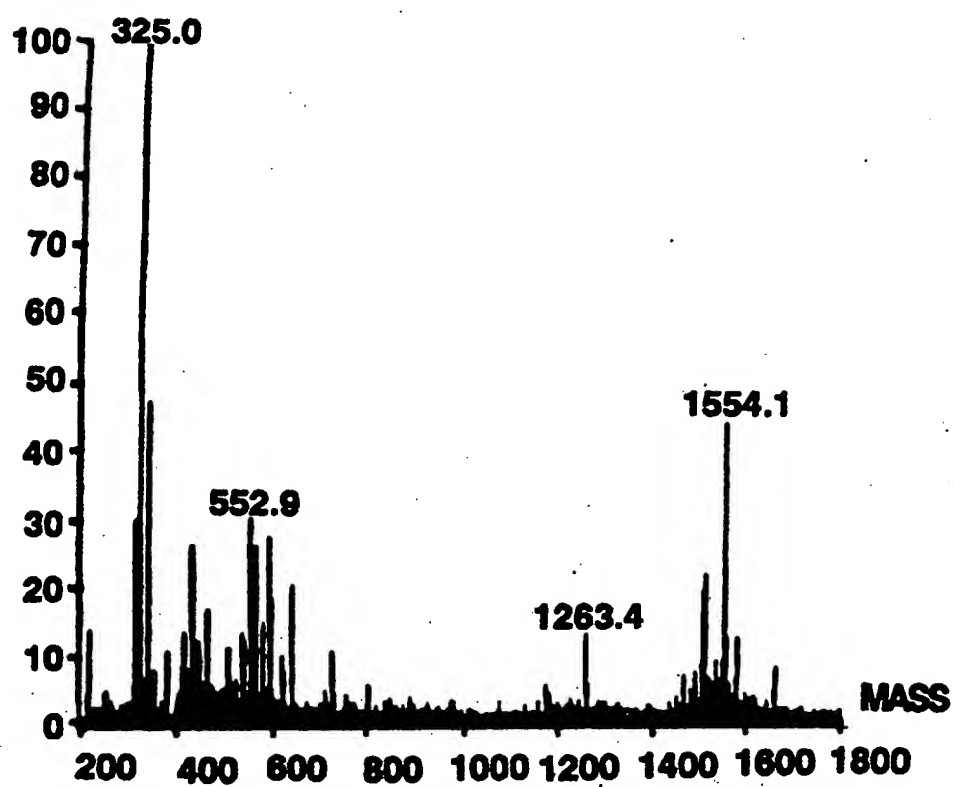


FIGURE 6B

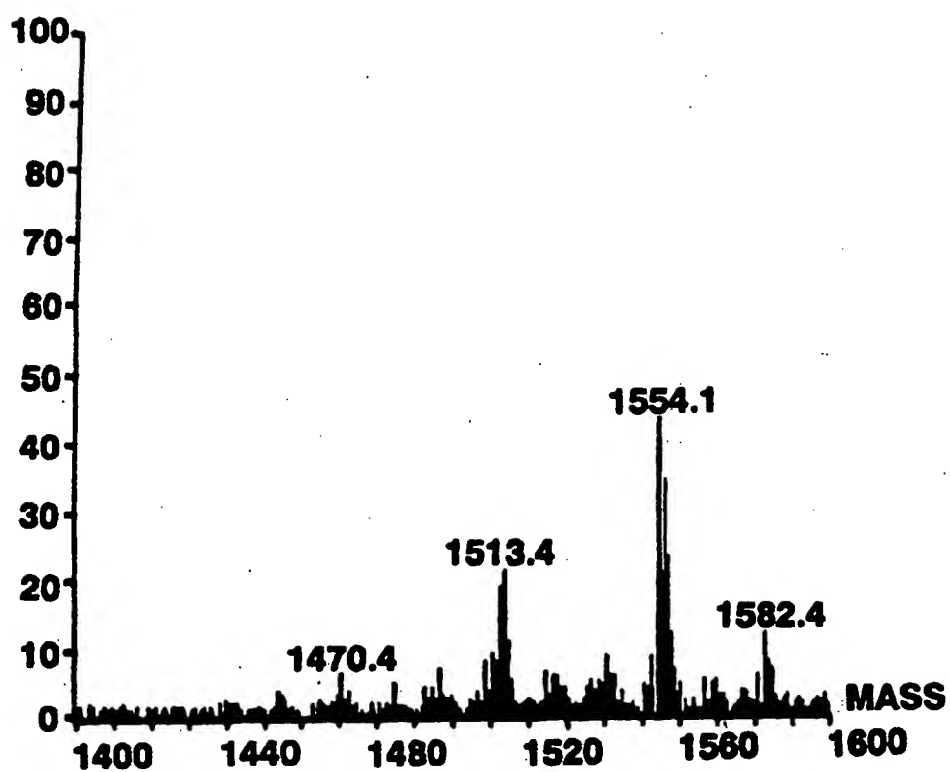


FIGURE 6C

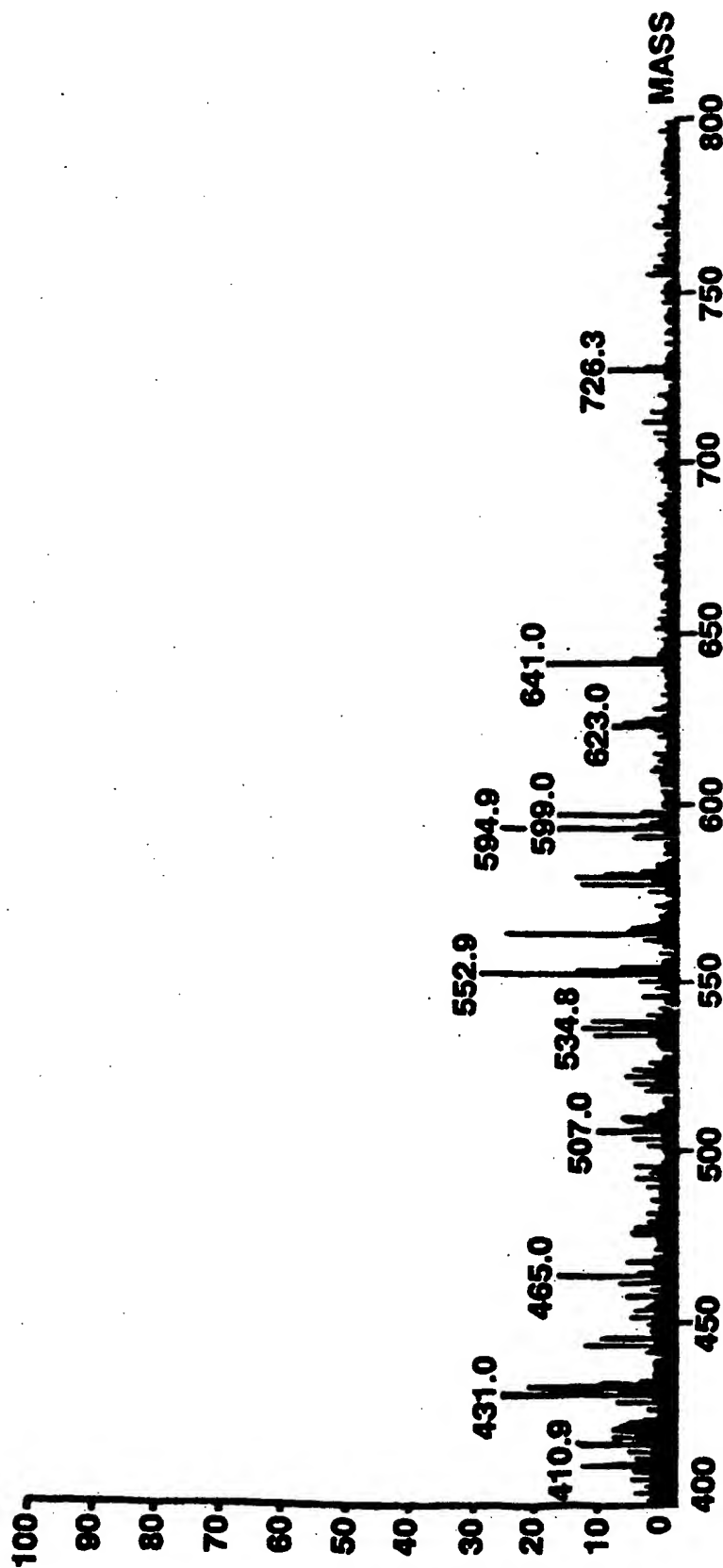
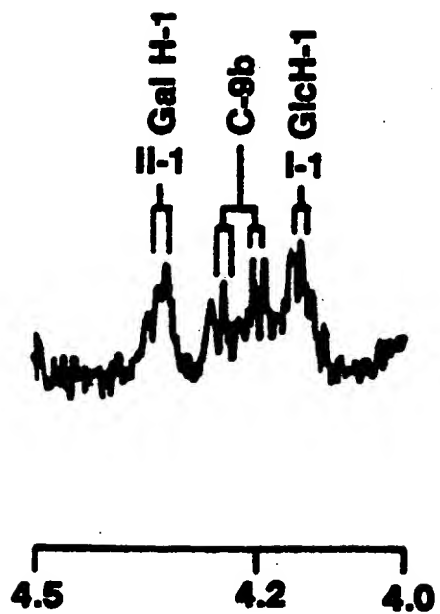


FIGURE 7



Partial 490 MHz, <sup>1</sup>H-NMR spectrum  
of F2 in DMSO-d<sub>6</sub>/D<sub>2</sub>O at 303°K (4.5-4.0 ppm)

## VACCINE FOR STIMULATING OR ENHANCING PRODUCTION OF ANTIBODIES AGAINST 9-O-ACETYL GD3

This invention was made with government support under Grant Numbers CA-40532 and CA-43971, National Cancer Institute, Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in the invention.

### BACKGROUND OF THE INVENTION

Throughout this application, various publications are referenced by arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed in this application.

Gangliosides are prominent cell-membrane components of melanoma and other tumors of neuroectodermal origin. Many different gangliosides have been described in melanoma cells. These include GM3, GM2, GM1, GD3, GD1A, GD1B, GD2, GT1B, 9-0 acetyl GD3, and GT3 (1-2). Four of these, GM2, GD2, GD3, and 9-0 acetyl GD3, have attracted special attention because of wide distribution on melanoma cells and limited distribution on cells outside the central nervous system (3-6). The importance of GD2 and GD3 has been suggested by regression of melanoma and neuroblastoma metastases in some patients treated with anti-GD3 and anti-GD2 monoclonal antibodies (MmAb) (7-9). Many patients have received vaccines containing melanoma cells expressing these gangliosides (10-13). These studies have shown that GM2 antibodies were frequently induced, antibodies against GD2 were occasionally induced, and antibodies against GD3 and 9-0 acetyl GD3 were never detected. Due to uncertainties about expression of each ganglioside in the vaccines, or on the target cells used for serological analysis, the relative immunogenicity of GM2, GD2, GD3 and 9-0 acetyl GD3 remained unknown.

In attempts to induce active immunity against melanoma, we focused on GM2 in initial studies because its distribution on cell lines (defined by anti-GM2 MmAb 5.3) and on various tissues (detected by extraction and thin layer chromatography) was quite restricted (3). In a series of experiments in the mouse, we have identified immunizing procedures that facilitate the serologic response to GM2 and other gangliosides (14-15): pretreatment with low dose cyclophosphamide and immunization with GM2 attached to adjuvant-carriers such as BCG or *Salmonella minnesota* mutant R595. Trials comparing these approaches in early stage melanoma patients demonstrated BCG to be a significantly better adjuvant than R595 and patients pretreated with a low dose of cyclophosphamide had significantly higher titers of anti-GM2 antibody than those not receiving this pretreatment (12). IgM antibodies were induced in 72% of patients receiving the BCG-GM2 vaccine, and these were capable of lysing human tumor cells in the presence of human complement. IgG antibodies were detected in 25% of the immunized patients. The pattern of primary and secondary antibody response to immunization was most consistent with GM2 acting as a T cell independent antigen.

In the study reported here, we apply the same immunization approach, CY + BCG - purified ganglioside, to test the immunogenicity of GD2, GD3, and 9-0-acetyl GD3. The relative immunogenicity of these gangliosides was examined in AJCC stage III and IV melanoma patients who were free of detectable disease after surgery. 9-0-acetyl GD3 is identified as a second effective immunogen.

Nothing yet is known about the immunogenicity of 0-acetyl-GD3. Although structurally close to GD3, the molecule is antigenically different (5,6 16-17). While Ravindranaths et al. have recently described 2 melanoma patients with antibody reactivity against 9-0-acetyl and/or 4-0 acetyl GD3, the incidence of such antibodies in melanoma patients was unknown (18). In this study we report our attempts at chemically synthesizing 0-acetyl-GD3 and characterizing the properties of the products obtained. As immunization of patients with malignant melanoma with these derivatives is envisaged, we examined their immunogenicity in the mouse, by analysing the humoral immune response after vaccination with 0-acetyl-GD3 derivatives.

### SUMMARY OF THE INVENTION

This invention provides vaccine for stimulating or enhancing in a subject to whom the melanoma vaccine is administered, production of antibodies against 9-0-acetyl GD3 ganglioside comprising an amount of a 9-0-acetyl GD3 ganglioside effective to stimulate or enhance antibody production in the subject and a pharmaceutically acceptable carrier.

This invention further provides the aforementioned melanoma vaccine which additionally comprises an adjuvant. In accordance with the teachings of this invention, the adjuvant may be a microbial adjuvant. This microbial adjuvant may further comprise *Salmonella minnesota* R595 or bacillus Calmette-Guerin.

This invention also provides the aforementioned melanoma vaccine wherein the effective amount of 9-0-acetyl GD3 ganglioside comprises an amount between about 50 micrograms and about 300 micrograms.

Additionally, this invention also provides the aforementioned melanoma vaccine wherein the 9-0-acetyl GD3 ganglioside is purified from a biological source. In accordance with the teachings of this invention the biological source may be melanoma cells, milk or buttermilk.

This invention also provides the aforementioned melanoma vaccine which additionally comprises purified GM2 ganglioside.

Furthermore, this invention also provides the aforementioned melanoma vaccines wherein the subject is afflicted with cancer and the antibody produced in the subject upon administration of the melanoma vaccine effectively treats the cancer. Additionally provided are the aforementioned melanoma vaccines wherein the subject is susceptible to cancer and the antibody produced in the subject upon administration of the melanoma vaccine effectively prevents the cancer. In accordance with the teachings of this invention, the cancer is of neuroectodermal origin, and the cancer of neuroectodermal origin may be a melanoma.

This invention further provides a method for stimulating or enhancing in a subject production of antibodies against 9-0-acetyl GD3 ganglioside comprising administering to the subject an effective dose of the aforementioned melanoma vaccines.



Additionally, this invention provides a method for treating cancer and a method for preventing cancer in a subject affected with cancer comprising administering to the subject an effective dose of the aforementioned melanoma vaccines. In accordance with the teachings of this invention, 9-O-acetyl GD3 ganglioside of the aforementioned melanoma vaccine methods may be bound to a microbial adjuvant. Furthermore, the 9-O-acetyl GD3 ganglioside may be bound to the microbial adjuvant by a hydrophobic bond between the lipid portion of the 9-O-acetyl GD3 ganglioside and the cell membrane of the microbial adjuvant and the microbial adjuvant may be *Salmonella minnesota* R595 or bacillus Calmette-Guerin.

This invention additionally provides that in the aforementioned melanoma vaccine methods, the cancer may be of neuroectodermal origin, and the cancer of neuroectodermal origin may be a melanoma. Furthermore, in the aforementioned melanoma vaccine methods, an effective amount of cyclophosphamide may be administered to the subject prior to administering the melanoma vaccine, and the cyclophosphamide also may be administered between about 3 days and about 7 days prior to the administering the melanoma vaccine. Also in accordance with the teachings of this invention, the effective amount of cyclophosphamide may be between about 1 mg/m<sup>2</sup> and about 500 mg/m<sup>2</sup>.

This invention additionally provides a 9-O-acetyl GD3 ganglioside designated F2 characterized by the presence of a second acetyl group and the mass spectra show in FIGS. 6A, 6B, and 6C. Also provided is a 9-O-acetyl GD3 ganglioside designated F3 characterized by the presence of 2 or more additional acetyl groups and recoverable from a mixture of acetylated derivatives of GD3 by a high pressure liquid chromatography. Further, the invention provides for a mixture of the 9-O-acetyl GD3 gangliosides F2 and F3.

Finally, the invention provides a melanoma vaccine for stimulating or enhancing in a subject to whom the melanoma vaccine is administered, production of antibodies against 9-O-acetyl GD3 ganglioside comprising an amount of a 9-O-acetyl GD3 ganglioside of any of F2 or F3 ganglioside or mixture thereof effective to stimulate or enhance antibody production in the subject and a pharmaceutically acceptable carrier.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the migration of O-acetylated derivatives of GD3 in TLC. C, O-acetylated GD3; St, reference gangliosides GM1 and GD3; HPTLC silica gel plate; running solvent: chloroform/methanol/0.2% aqueous CaCl<sub>2</sub> 60:35:8 v/v; spray reagent: orcinol/H<sub>2</sub>SO<sub>4</sub>.

FIG. 2 shows the separation of O-acetyl-GD3 derivatives by preparative HPLC. NH<sub>2</sub>-column (21.4×250 mm); buffer 1: acetonitrile/5mM phosphate 83:17 v/v; pH 5.6; buffer 2: acetonitrile/20mM phosphate 1:1 v/v; pH 5.6 programmed as follows: 30 min isocratic buffer 1/buffer 2 90:10 v/v; 90 min with a linear gradient from buffer 1/buffer 2 90:10 v/v to buffer 1/buffer 2 50:50 v/v; 30 min isocratic buffer 1/buffer 2 50:50 v/v; flow rate 9 ml/min; eluting gangliosides were monitored at 205 nm in a flow-through detector. F1-3 representing isolated fractions.

FIG. 3 shows the TLC-analysis of isolated O-acetyl-GD3 derivatives F1, F2 and F3 before and after base treatment. bt referring to base treated fraction; HPTLC silica gel plate; running solvent: chloroform/me-

thanol/0.2% aqueous CaCl<sub>2</sub> 60:35:8 v/v; spray reagent: orcinol/H<sub>2</sub>SO<sub>4</sub>.

FIG. 4 shows the immune reactivity of TLC-separated O-acetyl-GD3 derivatives F1, F2 and F3 before and after base treatment with mAbs R24 (recognizing GD3), D.1.1. and ME 311 (recognizing 9-O-acetyl GD3). HPTLC silica gel plate; running solvent: chloroform/methanol/0.2% aqueous CaCl<sub>2</sub> 60:35:8 v/v; detection: peroxidase and 4-chloro-1-naphthol staining.

FIG. 5 shows the TLC-analysis of neutral core products obtained after exoglycosidase treatment of fraction F1. Lanes: 1, F1+sialidase; 2, F1+sialidase followed by base treatment; 3, F1+sialidase followed by  $\beta$ -galactosidase; 4, F1+sialidase +  $\beta$ -galactosidase followed by base treatment; 5, neutral glycosphingolipids derived from human spleen; 6, GD3 +sialidase; 7, GD3 +sialidase followed by base treatment; 8, GD3 +sialidase followed by  $\beta$ -galactosidase; 9, GD3 +sialidase +  $\beta$ -galactosidase followed by base treatment; HPTLC silica gel plate; running solvent: chloroform/methanol/water 65:25:4 v/v; spray reagent: orcinol/H<sub>2</sub>SO<sub>4</sub>.

FIG. 6 shows the FAB-MS spectra of fraction F2.

A: complete spectrum;

B: partially extended spectrum mass range 1400-1600; and

C: partially extended spectrum mass range 400-800.

FIG. 7 shows the partial 490-MHz <sup>1</sup>H NMR spectrum of fraction F2 in DMSO-d<sub>6</sub>/D<sub>2</sub>O at 303° K. (4.5-4.2 ppm) showing the chemical shifts between H-1 and I-1 characteristic for 9-O-acetylation of sialic acids.

#### DETAILED DESCRIPTION OF THE INVENTION

This invention provides a melanoma vaccine for stimulating or enhancing in a subject to whom the vaccine is administered, production of antibodies against 9-O-acetyl GD3 ganglioside comprising an amount of a 9-O-acetyl GD3 ganglioside effective to stimulate or enhance antibody production in the subject and a pharmaceutically acceptable carrier.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents.

In this method, the administration of the compound may be effected by any of the well known methods, including but not limited to intravenous, intramuscular, and subcutaneous administration.

This invention further provides the aforementioned melanoma vaccine which additionally comprises an adjuvant. In accordance with the teachings of this invention, the adjuvant may be a microbial adjuvant. This microbial adjuvant may further comprise *Salmonella minnesota* R595 or bacillus Calmette-Guerin.

A range of the amount of *Salmonella minnesota* R595 may be employed. However, the preferred amount present in the vaccine is an amount between about 0.2 mg and about 1.5 mg. Further, a range of the amount of bacillus Calmette-Guerin may be employed. However, the preferred amount present in the vaccine is an amount between about 10<sup>5</sup> viable units and about 3×10<sup>7</sup> viable units.

This invention also provides the aforementioned melanoma vaccine wherein the effective amount of 9-O-acetyl GD3 ganglioside comprises an amount between about 50 micrograms and about 300 micrograms.

Additionally, this invention also provides the aforementioned melanoma vaccine wherein the 9-0-acetyl GD3 ganglioside is purified from a biological source. In accordance with the teachings of this invention the biological source may be melanoma cells, milk or buttermilk.

This invention also provides the aforementioned melanoma vaccine which additionally comprises purified GM2 ganglioside.

Furthermore, this invention also provides the aforementioned melanoma vaccines wherein the subject is afflicted with cancer and the antibody produced in the subject upon administration of the vaccine effectively treats the cancer. Additionally provided are the aforementioned melanoma vaccines wherein the subject is susceptible to cancer and the antibody produced in the subject upon administration of the vaccine effectively prevents the cancer. In accordance with the teachings of this invention, the cancer is of neuroectodermal origin, and the cancer of neuroectodermal origin may be a melanoma.

This invention further provides a method for stimulating or enhancing in a subject production of antibodies against 9-0-acetyl GD3 ganglioside comprising administering to the subject an effective dose of the aforementioned melanoma vaccines.

Additionally, this invention provides a method for treating cancer and a method for preventing cancer in a subject affected with cancer comprising administering to the subject an effective dose of the aforementioned melanoma vaccines. In accordance with the teachings of this invention, 9-0-acetyl GD3 ganglioside of the aforementioned melanoma vaccine methods may be bound to a microbial adjuvant. Furthermore, the 9-0-acetyl GD3 ganglioside may be bound to the microbial adjuvant by a hydrophobic bond between the lipid portion of the 9-0-acetyl GD3 ganglioside and the cell membrane of the microbial adjuvant and the microbial adjuvant may be *Salmonella minnesota* R595 or bacillus Calmette-Guerin. In the case of the melanoma vaccine which additionally comprises purified GM2 ganglioside, either the 9-0-acetyl GD3 or GM2 ganglioside may be bound to the adjuvant.

This invention additionally provides that in the aforementioned melanoma vaccine methods, the cancer may be of neuroectodermal origin, and the cancer of neuroectodermal origin may be a melanoma. Furthermore, in the aforementioned melanoma vaccine methods, an effective amount of cyclophosphamide may be administered to the subject prior to administering the melanoma vaccine, and the cyclophosphamide also may be administered between about 3 days and about 7 days prior to the administering the vaccine. Also in accordance with the teachings of this invention, the effective amount of cyclophosphamide may be between about 1 mg/m<sup>2</sup> and about 500 mg/m<sup>2</sup>.

This invention additionally provides a 9-0-acetyl GD3 ganglioside designated F2 characterized by the presence of a second acetyl group and the mass spectra show in FIGS. 6A, 6B, and 6C. Also provided is a 9-0-acetyl GD3 ganglioside designated F3 characterized by the presence of 2 or more additional acetyl groups and recoverable from a mixture of acetylated derivatives of GD3 by a high pressure liquid chromatography. Further, the invention provides for a mixture of the 9-0-acetyl GD3 gangliosides F2 and F3.

Finally, the invention provides a melanoma vaccine for stimulating or enhancing in a subject to whom the

vaccine is administered, production of antibodies against 9-0-acetyl GD3 ganglioside comprising an amount of a 9-0-acetyl GD3 ganglioside of any of F2 or F3 ganglioside or mixture thereof effective to stimulate or enhance antibody production in the subject and a pharmaceutically acceptable carrier.

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

## EXPERIMENTAL DETAILS

### Materials and Methods

**Patients.** Melanoma patients with regional skin and lymph node metastases or systemic metastases were considered eligible if tumors had been resected within four months and if they were free of detectable melanoma. None of the patients had received chemotherapy or radiation therapy within eight weeks. Patients were examined at six-week intervals. Chest x-rays, liver function tests, and urinalysis were performed at three-month intervals. Blood for serologic tests was obtained at two-week intervals.

**Gangliosides.** GM2 and GD2 were prepared by treating GM1 and GD1B with  $\beta$ -galactosidase (G. W. Jourdan, University of Michigan, Ann Arbor, Mich.) according to published methods (19). GM1, GD1A, and GT1 were purchased from Supelco (Bellafonte, PA). GD1B was purchased and GD3 (for conversion to 9-0-acetyl GD3) was kindly supplied as a gift by Fidia Research Labs, Abano Terme, Italy. 9-0-acetyl GD3 was prepared by acetylation of GD3 as previously described. GM3 and GD3 (for GD3 vaccines) were purified from dog erythrocytes and human melanoma respectively as previously described (3).

**Serological Procedures.** The enzyme-linked immunosorbent assay (ELISA) was performed with rabbit anti-human IgM, anti-human IgG, or protein A conjugated to alkaline phosphatase (Zymed Laboratories, San Francisco) (12). Normal or pretreatment sera served as blanks and were subtracted from experimental values. Antibody titer was defined as the highest serum dilution yielding a corrected OD > 0.190. Reagents for dot blot immune stains were peroxidase conjugated goat anti human IgM and goat antihuman IgG (Tage, Burlingame, CA) diluted 1:500. Dot blots were graded as negative, 1+, 2+ or 3. Complement dependent cytotoxicity assays were performed with normal human serum (diluted 1:3) as the complement source as previously described (11).

**Skin Tests for Delayed Hypersensitivity (DTH).** Six, 12, and 25 mg of the immunizing ganglioside was suspended in 0.05 ml normal saline and injected intradermally. Skin tests for DTH against mumps and other recall antigens were performed and interpreted as previously described (20).

**Ganglioside Vaccines.** 10<sup>7</sup> viable units of BCG (Tice strain, University of Illinois), or 3 × 10<sup>6</sup> units in the case of patients showing strong reactions to BCG, were suspended in distilled water by sonication and added to tubes containing 50 mg of 9-0 acetyl GD3, 100, 200 or 300 µg of GM2, 200 µg of GD2, 300 µg of GD3, or 200 µg of GM2, GD2 and GD3. The suspension was lyophilized and suspended in PBS shortly before vaccine administration. Patients received three vaccinations

intradermally at two-week intervals on a rotating basis to uninvolved extremities. In addition, a booster immunization was administered two months after the third vaccination.

Cycl phosphamide Administration. Cy (Cytosan, Mead Johnson and Co., Evansville, Ind.) 200 mg/M was administered to all patients IV four to six days prior to the initial immunization.

Glycolipids and chemicals: GM3, GM2 and GM1 gangliosides were purchased from, and GD3 was generously provided by Fidia Research Laboratories (Abanc Terme, Italy); neutral glycosphingolipids were prepared from human spleen in our laboratory by published procedures (21); HPTLC silica gel plates were obtained from E. Merck (Darmstadt, FRG); Nitrocellulose membranes (0.2  $\mu$ m) were obtained from Schleicher and Schuell, Inc. (Keene, NH); Preparative (21.4 $\times$ 250 mm) and semi-preparative (10 $\times$ 250 mm) aminopropyl and C18 HPLC-columns were obtained from Rainin Instruments Co. (Ridgefield, N.J.); analytical (3.9 $\times$ 300 mm) aminopropyl HPLC-column and Sep-Pak C18-cartridges were obtained from Waters Associates (Milford, Mass.); DEAE-Sephadex A25 4-chloro-1-naphthol, p-nitrophenyl phosphate disodium, sodium-taurodeoxycholate and N-acetyl-imidazole were obtained from Sigma Chemical Co. (St. Louis, Mo.). Cyclophosphamide (Cytosan) was obtained from Mead Johnson (Syracuse, N.Y.).

Enzymes. Endoglycoseramidase was kindly provided by Dr. Makoto Ito from the Mitsubishi-Kasei Institute of Life Science (Tokyo, Japan); *V. cholerae* sialidase (E.C. 1.2.1.13) was obtained from Calbiochem-Behring Corporation (La Jolla, CA). Jack bean  $\beta$ -galactosidase (E.C. 3.2.1.23) was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Monoclonal antibodies (mAbs): mAbs rabbit anti-mouse conjugated with horseradish peroxidase for ITLC was obtained from Dako Corporation (Santa Barbara, Calif.); mAbs rabbit anti mouse IgM or IgG conjugated with horseradish peroxidase or alkaline phosphatase were obtained from Zymed (San Francisco, Calif.); mAb D.1.1. was kindly provided by Dr. David A. Cheresh (Scripps-Clinic, La Jolla, CA, ref. 5); mAb ME 311 was provided by Dr. Jan Thurin (The Wistar Institute, Philadelphia, Pa., ref. 6) and Jones antibody was provided by Dr. Andrew S. Blum (Rockefeller University, New York, N.Y., ref. 22). mAbs R 24, C5 and K9 were generated in our laboratory (23).

Synthesis of 0-acetyl-GD3 derivatives: GD3 was 0-acetylated slightly modified according to Haverkamp, et al. (24). 10 mg GD3, well dried, were dissolved in 250  $\mu$ l pyridine (water free) in a small reaction vessel and incubated at 50° C. After 30 min. 150  $\mu$ l N-acetyl-imidazole in pyridine (10  $\mu$ g/ $\mu$ l) were added and the mixture was incubated at 50° C. At different time intervals, aliquots were taken and monitored by TLC and by analytical HPLC. Usually after 70h, more than 90% of GD3 was converted and the reaction was stopped by evaporating under a stream of nitrogen. Toluene was added during evaporation to remove remaining pyridine.

High Performance Thin Layer Chromatography (HPTLC): TLC-analysis was performed on HPTLC silica gel plates, whereby gangliosides and ganglioside-derivatives were run in solvent system chloroform/methanol/1/0.2% aqueous  $\text{CaCl}_2$  60:35:8 (v/v), neutral glycosphingolipids in solvent system chloroform/methanol/water 65:25:4 (v/v), ceramides in chloroform-

/methanol 95:5 (v/v) (25) and oligosaccharides in ethanol/n-butanol/pyridine/water/glacial acetic acid 100:10:10:30:3 (v/v) (26). Gangliosides, ganglioside derivatives and oligosaccharides were visualized with orcinol/ $\text{H}_2\text{SO}_4$  or resorcinol/ $\text{HCl}$ , neutral glycosphingolipids with orcinol/ $\text{H}_2\text{SO}_4$  and ceramides in i dine vapor and Coomassie Blue (27).

High Performance Liquid Chromatography (HPLC): HPLC was performed using a Waters computer operated liquid chromatography system (Model 501). To monitor GD3 conversion and to estimate approximate yields, analytical HPLC on a  $\text{NH}_2$ -column (3.9 $\times$ 300 mm) was performed as described by Gazzotti, et al. (28). For preparative separation  $\text{NH}_2$ -columns (21.4 $\times$ 250 mm) were used and aliquots of 5 mg ganglioside dissolved in distilled water were injected. The above mentioned method was modified as follows:

Buffer 1: acetonitrile/5mM phosphate 83:17 (v/v), pH 5.6; buffer 2: acetonitrile/20mM phosphate 1:1 (v/v), pH 5.6, programmed as follows: 30 min isocratic buffer 1/buffer 2 90:10 (v/v); 90 min. with a linear gradient from buffer 1/buffer 2 90:10 (v/v), to buffer 1/buffer 2 50:50 (v/v); 30 min isocratic buffer 1/buffer 2 50:50 (v/v) followed by 30 min buffer 2 100%; flow rate was 9 ml/min; eluting gangliosides were monitored at 205 nm in a flow-through detector. Fractions were pooled, dried on a rotary evaporator and re-chromatographed in the same system, followed by desalting on a C18-column (10 $\times$ 250 mm) using 200 ml water, 200 ml water/methanol 1:1 (v/v) and 200 ml methanol. Flow rate was 4 ml/min. Methanol fractions were dried and homogeneity was monitored by TLC.

Enzyme hydrolysis: 0-acetyl GD3 derivatives were treated with *V. cholerae* sialidase (29), neutral cleavage products were separated by DEAE-Sephadex chromatography (30) and analyzed by TLC before and after base treatment with 0.05 M NaOH in methanol for 1 h at 37° C. In the case of sequential hydrolysis analysis, gangliosides were first cleaved by *V. cholerae* sialidase; following purification by Sep-Pak C18 chromatography (31) and separation on DEAE Sephadex A 25 the neutral fractions were subsequently treated with  $\mu$ -galactosidase. The reaction mixture contained 10–20  $\mu$ g glycolipid, 0.1% sodium taurodeoxycholate dissolved in 0.05 M citric acid-sodium citrate buffer pH 4.0 and 0.1 U  $\beta$ -galactosidase (32). Incubation was carried out at 37° C. for 24 h. Following removal of sodium taurodeoxycholate by DEAE Sephadex A 25, aliquots of the cleaved glycolipids were analyzed by TLC before and after base treatment in solvent system chloroform/methanol/water 65:25:4 (v/v).

Endoglycoseramidase treatment of 0-acetyl GD3 derivatives was performed according to Ito, et al. (25). Oligosaccharides and ceramides were analyzed by TLC before and after base treatment. Susceptibility of 0-acetyl GD3 derivatives to cleavage by serum esterase activity was analyzed as follows: 20  $\mu$ g GD3 or GD3 derivatives were incubated in the presence of 50  $\mu$ l fresh human or mouse serum or in PBS containing 2% BSA for 24 h at 37° C. The glycolipids were re-extracted with chloroform/methanol/water 10:10:1 (v/v), purified by Sep-Pak C18 chromatography and analyzed by TLC and ITLC with mAbs R24 and D.1.1.

Reduction of the double bond in the ceramide portion of GD3 was performed with sodium borohydride and palladiumchloride as catalyst (21).

Negative ion fast atom bombardment (FAB) mass spectrometry: Negative ion FAB mass spectra were

recorded on a VG Analytical (model ZAB-SE) high resolution mass spectrometer (Manchester, England) equipped with a xenon ion source. The FAB ion source was typically maintained at 8KV with 1 mA current. The sample was dissolved in a small amount of chloroform/methanol 2:1 (v/v) and an aliquot containing about 10 µg of the sample was placed on the stainless steel sample holder. Thioglycerol, about 2 µl, was added as the matrix solvent before analysis.

High resolution nuclear magnetic resonance spectroscopy (NMR): For NMR analysis fraction F2 was further purified by Sephadex LH-20 chromatography, purged of exchangeable protons by dissolving in DMSO-d<sub>6</sub>/D<sub>2</sub>O 1:1 (v/v) and immediately lyophilized. The sample was then dissolved in DMSO-d<sub>6</sub>/D<sub>2</sub>O 98:2 (v/v) containing traces of tetramethylsilane as internal standard. NMR spectra were obtained with a Bruker WM 500 spectrometer equipped with an Aspect 2000 computer operating in the Fourier transform mode as previously described (33).

Animals: Female BALB/c-057BL/6 FI-mice obtained from Jackson Laboratory (Bar Harbor, ME) 6 weeks of age were used for vaccination.

Vaccination: Mice were pretreated with cyclophosphamide at a dose of 15 mg/kg intraperitoneal 3 days before the first vaccine. Vaccines were prepared as follows: GD3 or GD3-derivatives prior dried down in conical tubes, were resuspended in dest. water containing *Salmonella minnesota* mutant R 595, prepared as described (15). The mixture was lyophilized and emulsified in PBS prior to vaccine administration. Groups of 10 mice, randomly selected, were immunized subcutaneously with given vaccine twice two weeks apart. Vaccines contained each 10 µg glycolipid and 0.5 mg *S. minnesota* R 595 in a total volume of 100 µl PBS.

Mice were bled from the retro-orbital sinus before and two weeks after the first and second vaccine. Serum samples for serological testings were stored at -20° C. Sera were analyzed by dot plot assays, ELISA and ITLC. Dot plot immune stains were performed as previously described (34) modified as follows: 0.20 µg glycolipid was spotted on nitrocellulose stripes. The stripes were blocked for 1 h at room temperature in PBS containing 5% FCS, 1% BSA and 0.1% sodium acid and incubated with mouse serum (1:150) in small trays overnight at room temperature. After washing 5x in PBS containing 0.05% Tween 20, stripes were reacted with HRP-conjugated anti mouse IgM or IgG antibodies (1:200) for 5h at room temperature. Peroxidase staining was performed as described for ITLC. Stains were quantitated as negative, 1+, 2+ and 3+.

ELISA: 0.1 µg glycolipid in 50 µl ethanol was dried per well in a 96 well plate and blocked with PBS containing 3% BSA 2 h at 37° C. Wells were incubated with mouse sera serially diluted with PBS containing 3% BSA for 1h at room temperature. Plates were washed 5x with PBS containing 0.05% Tween 20 and incubated with AP-conjugated anti mouse IgM or IgG antibody (1:200) for 1 h at room temperature. After 5x washing with the above mentioned buffer, 100 µl phosphatase substrate solution (0.2% p-nitrophenyl phosphate disodium in PBS containing 3% BSA) was added to each well. After 30 min absorbance of the reaction product was measured at 414 nm. To eliminate the effect of unspecific "sticky" sera, sera was also tested on plates which had been processed identically but to which n

glycolipid had been added. The optic densities at each titer obtained on these plates was subtracted from the experimental value, yielding a corrected optic density.

ITLC: Immunostaining of gangliosides and ganglioside derivatives with monoclonal antibodies or mouse sera after separation on HPTLC silica gel glass plates was performed slightly modified according to Magnani (35) as previously described (36).

#### Serologic Response of Vaccinated Patients

Vaccination with BCG-ganglioside vaccines was well tolerated; all side effects detected were attributed to BCG. Vaccines resulted in low grade fever (less than 39° Centigrade) and prominent ulceration or inflammation at vaccine sites, requiring a decrease in the BCG dose to 3 × 10<sup>6</sup> organisms in 65% of patients. No neurologic or other detectable abnormalities were seen. Skin tests for delayed hypersensitivity to the immunizing gangliosides and to recall antigens were performed. Most patients were reactive with at least one recall antigen, but none were reactive with the immunizing ganglioside.

Table 1 shows the results of ELISAs for antibodies against the four gangliosides in sera from normal individuals and unvaccinated and vaccinated melanoma patients. Spontaneous high titer antibodies (greater than 1/40) against GD2 and GD3 were not detected in sera from normal donors or untreated melanoma patients. One normal donor had a titer of 1/80 against GM2 and another a titer of 1/80 against 9-0 acetyl GD3. Both were confirmed to be specific in immune stains. Several low titer antibody responses were detected against GM2 and 9-0-acetyl GD3. These sera failed to react in dot blot immune stains, so the specificity of these antibodies could not be analyzed. The 44 patients immunized with Cy+BCG-GM2 represent a composite of 24 previously reported patients and 20 patients immunized more recently. Seventy-five per cent of patients produced a high titer GM2 antibody response (see Table 1). The four additional patients with anti-GM2 titers of 40 all gave clear reactions in immune stains, reacting with GM2 and no other gangliosides. Of the seven patients with titers of 20 or less, only three received the full series of vaccinations. Recurrent melanoma was detected in the other four patients during the course of immunization, requiring treatment with other modalities before the booster immunization could be administered. Hence 92% of patients receiving the full series of immunizations produced anti GM2 antibodies. High titer antibody responses against GD2 and GD3 were not seen in patients immunized with either GD2 or GD3, or the six patients immunized with three gangliosides. Four patients produced anti GD2 titers of 40, two of these showed specific anti GD2 reactivity in immune stains. 9-0-acetyl GD3 was highly immunogenic, producing high titer antibodies in all six patients immunized. Sequential antibody responses detected in ELISAs on 9-0-acetyl GD3 for these six patients is shown in FIG. 1. While three of the patients produced high titer antibody during the initial series of immunizations, all showed the highest titer after the fourth (booster) immunization, a result similar to that seen with immunization against GM2. Also as described for GM2, the high titer antibody response was short-lived, median duration 8 weeks.

TABLE 1

ANTIBODY TITERS (ELISA) OF NORMAL DONORS, UNTREATED MELANOMA PATIENTS AND MELANOMA PATIENTS AFTER IMMUNIZATION WITH PURIFIED GANGLIOSIDE VACCINES								
Treatment	Total No. Patients	Target	ELISA RESULTS* No. of Patients with a Given Titer					
			0	20	40	80	160	320
Untreated	44	GM2	37	4	2	1	0	0
Normal Donors	30	GD2	29	1	0	0	0	0
	30	GD3	30	0	0	0	0	0
	30	9-O-acetyl GD3	25	3	1	1	0	0
Melanoma Patients	48	GM2	37	8	3	0	0	0
	30	GD2	28	2	0	0	0	0
	30	GD3	30	0	0	0	0	0
	30	9-O-acetyl GD3	22	5	3	0	0	0
Vaccinated With								
BCG-GM2	44	GM2	4	3	4	14	11	8
BCG-GD2	6	GD2	2	2	2	0	0	0
BCG-GD3	6	GD3	4	2	0	0	0	0
BCG-GM2 + GD2 + GD3	6	GM2	1	1	1	3	0	0
BCG-GM2 + GD2 + GD3		GD2	1	3	2	0	0	0
BCG-GM2 + GD2 + GD3		GD3	3	3	0	0	0	0
BCG-9-O-acetyl GD3	6	9-O-acetyl GD3	0	0	0	1	1	4

\*Results on GM2 of normal donors and untreated melanoma patients published previously - ref. 10, and of 24 of the 44 GM2 vaccinated patients - ref. 11.

#### Specificity Analysis of Reactive Sera

The specificity of GM2-reactive sera induced by Cy+BCG-GM2 has been analyzed in detail and reported previously. Reactivity is restricted to GM2 and N-glycolyl GM2. Analysis of sera from the additional twenty patients reported here by dot blot immune stains and inhibition assays confirms this finding. Reactivity was restricted to GM2 and N-glycolyl GM2 in all patients. Dot blot immune strains with post-immunization sera from patients receiving BCG/GD2 and BCG/GD3 vaccines showed a low level of reactivity against GD2 in the two patients immunized with GM2+GD2+GD3 and having GD2 titers by ELISA of 40. Reactivity was restricted to 9-O-acetyl GD3. Inhibition assays were used to look with greater precision for cross reactivity of these antibodies with GD3 and none was detected.

#### Immune Adherence Assay of 9-O Acetyl GD3 Antibodies

In preliminary studies, reactivity against melanoma cells has been shown. The titers in six patients ranged from  $\frac{1}{4}$  to 1/64.

Preparation of the O-acetyl derivative of GD3, F1, F2 and F3:

GD3 was O-acetylated using N-acetyl-imidazole in pyridine. TLC-analysis, monitoring the process of acetylation, revealed that during the initial 30 h one major product was formed, migrating between GD3 and GM1, while the yield of higher migrating products remained low. After 70 h no further decrease of GD3, used as starting material, was observed and the ratio of all conversion products remained stable (FIG. 1). The conversion products were separated by preparative HPLC and pooled into 3 fractions referred to as F1, F2 and F3, respectively (FIG. 2). When the average ratio of the products (percent of total conversion products of four conversions) obtained from GD3 was estimated by densitometry after TLC separation or integration of the peak area obtained after analytical HPLC, F1 yielded 45  $\pm$  10%, F2 20  $\pm$  5%, F3 25  $\pm$  10% and GD3 10  $\pm$  7%.

Characterization of F1, F2 and F3:

The fractions obtained were characterized by TLC-analysis before and after base treatment (FIG. 3). Before base treatment, F1 migrated between GM1 and GD3, F2 ran slightly faster than GM1, and the 2 major bands of F3 migrated between GM2 and GM3. All fractions were sensitive to base treatment and were reconverted to a product co-migrating with the GD3, from which they were derived. Immune reactivity of the fractions with antiganglioside monoclonal antibodies was investigated by ITLC (Table 2).

GD3 and F1 showed reactivity only with anti GD3 antibodies, whereas F2 and F3 reacted with monoclonal antibodies against 9-O-acetyl GD3 as well as GD3. This reactivity with antibodies against 9-O-acetyl GD3 was lost after base treatment (FIG. 4).

Further information about the localization of the acetyl groups was obtained by TLC-analysis of oligosaccharides and ceramides after treatment with endoglycoceramidase. Base treated and non base treated ceramides from F1, F2 and F3 co-migrated with ceramide derived from native GD3. Comparing the conversion products of original GD3 and GD3, in which the double bond in the ceramide proton was reduced prior to acetylation differences in quality and quantity of the bands could not be observed by TLC analysis, also indicating that the acetylation site was not in the ceramide moiety. Oligosaccharides, however, showed different mobility, with relative migration rates similar to that of their parent gangliosides and after base treatment they co-migrated with GD3-oligosaccharide, suggesting O-acetylation on the oligosaccharide moiety of GD3.

TABLE 2  
IMMUNOREACTIVITY OF O-ACETYL-GD3  
DERIVATIVES\*

DERIVATIVE	MABS					
	anti-9-O-AcGD3			anti-GD3		
	D.1.1 <sup>1</sup>	ME 311 <sup>2</sup>	JONES <sup>3</sup>	R24 <sup>4</sup>	C5 <sup>4</sup>	K9 <sup>4</sup>
GD3	-	-	-	+	+	+
F1	-	-	-	+	+	+
F2	+	+	+	+	+	+

TABLE 2-continued

IMMUNOREACTIVITY OF O-ACETYL-GD3 DERIVATIVES*						
DERIVATIVE	MABS					
	anti-9-O-AcGD3			anti-GD3		
	D.1.1 <sup>1</sup>	ME 311 <sup>2</sup>	JONES <sup>3</sup>	R24 <sup>4</sup>	C5 <sup>4</sup>	K9 <sup>4</sup>
F3	+	+	+	+	+	+

(<sup>1</sup>Cheresh, et al. 1984; <sup>2</sup>Thurin, et al. 1985; <sup>3</sup>Blum, et al. 1987; <sup>4</sup>Dippold, et al. 1980, \*tested by ITLC)

Immune reactivity of O-acetyl-GD3 derivatives F1, F2 and F3 with anti ganglioside monoclonal antibodies as determined by ITLC. mAbs were incubated at 4° C. overnight; mAb dilutions: K9, C5 and R24 20 Ig/ml; D.1.1. 1:500; Jones antibody 1:200; ME 311 supernatant 1:5; HRP-rabbit-anti-mouse 1:200.

TLC-analysis after treatment with *V. cholerae* sialidase revealed that the major hydrolysis product derived from F1 migrated between CMH and CDH, the majority of F2 comigrated with CDH derived from parent

tion F2 contains two O-acetyl groups and was O-acetylated in position C-9 of the sialic acid moiety and fraction F3 was probably poly-O-acetylated, containing various epitopes recognized by 9-O-acetyl GD3 specific antibodies.

Susceptibility of F1, F2 and F3 to cleavage by serum esterases:

Because immunization of melanoma patients with these O-acetyl GD3 derivatives was contemplated, it was important to determine whether they were resistant or susceptible to cleavage by serum esterases. After in vitro exposure to fresh human serum for 24 h at 37° C., followed by re-extraction, TLC-separation and chemical or immuno staining, all O-acetylated GD3 derivatives were recovered.

Immunogenicity of F1, F2 and F3 in mice:

Table 3 shows the results of vaccination studies.

TABLE 3

VACCINE	NO. OF MICE	TARGET	ELISA TITERS		DOT BLOT IMMUNE STAIN
			IgM	IgG	
GD3	10	GD3	40(2), 20(8)	—	(2)
		F1	—	—	
		F2	—	—	
		F3	—	—	
F1	10	GD3	80, 40, 20(4)	—	(3)
		F1	160, 40, 20(5)	—	(4)
		F2	80, 20(6)	—	(4)
		F3	—	—	(2)
F2	10	GD3	80, 40(4), 20(5)	—	(3)
		F1	—	—	(9)
		F2	80(2), 40(7), 20	—	(9)
		F3	80, 40(5), 20(2)	—	(9)
F3	10	GD3	40(2), 20(4)	—	(1)
		F1	—	—	(1)
		F2	320(2), 160(3), 40(3), 20(1)	1280(3), 660(2), 80(1)	
		F3	1280(2), 640(3), 160(3), 80(2)	1280(2), 640(3), 320, 160(2), 40, 20	

GD3, and the hydrolysis products of F3 co-migrated with CDH and the F1 hydrolysis product. Only bands co-migrating with CDH could be detected after base treatment. Sequential hydrolysis of fraction F1 first by sialidase followed by treatment with  $\beta$ -galactosidase and TLC analysis of the neutral cleavage products, before and after base treatment, resulted in the following: (FIG. 5). The neutral F1-hydrolysis product obtained after sialidase treatment, which migrated between CMH and CDH was resistant to  $\beta$ -galactosidase treatment, while the hydrolysis product co-migrating the CDH was completely converted to CMH.

Negative-ion fast atom bombardment mass spectroscopy of fraction F2 showed a highest peak of m/z 1554, corresponding to GD3 containing two acetyl-groups (1470 + 42 + 42). Fragments of m/z 1513 (GD3 + acetyl), m/z 1470 (GD3), m/z 641 (sialic acid + sialic acid + acetyl) and m/z 599 (sialic acid + sialic acid) could be detected indicating one acetylation site in the sialic acid portion of the GD3 molecule (FIG. 6A, 6B, and 6C).

This fraction was further analyzed by proton nuclear magnetic resonance spectroscopy to pin point the exact position of the O-acetylation site. The NMR spectrum of fraction F2 revealed typical chemical shifts in the range of 4.2 ppm which can not be detected in original GD3, indicating O-acetylation in position C9 of the sialic acid portion of GD3 (FIG. 7).

These observations suggest that fraction F1 was O-acetylated on the lactose core of the ganglioside, frac-

## DISCUSSION

We have studied sera and monoclonal antibodies derived from melanoma patients to identify melanoma cell surface antigens that are immunogenic in man (37-39). Since reactivity against melanoma antigens was found in only a small percentage of patients, we have attempted to induce antibodies using vaccines containing irradiated cells expressing them. While we have detected reactivity against a variety of antigens in these studies, reactions against gangliosides have been predominant (10-12). These have included serum antibodies against GM2 and GD2 and human monoclonal antibodies against GM2, GM3, GD2, GD3 and X. Likewise Tai et al. have reported that antibodies against GM2 were induced in 10 of 26 patients receiving irradiated whole melanoma cell vaccines and that antibodies against GD2 were detected in 2 patients (13). In a previous study, we used the immunization approach found most active in initial preclinical and clinical trials, Cy pretreatment to decrease suppressor activity and immunization with BCG conjugated to purified GM2 ganglioside. High titer antibody against GM2 was induced in 17 of 24 patients entered, 17 of 21 patients receiving the full course of immunizations. Since GM3 and GD3 are the most abundantly expressed gangliosides on most melanoma cells and no antibodies against these two have been detected, it has been assumed that they are



not immunogenic. Nothing was known about the immunogenicity of 9-0-acetyl GD3. While Ravindranaths et al. have recently described 2 melanoma patients with antibody reactivity against 9-0-acetyl and/or 4-0 acetyl GD3, the incidence of such antibodies in melanoma patients was unknown (18). No antibodies against 9-0-acetyl GD3 have been reported in patients receiving whole cell melanoma vaccines, but this may reflect lack of 9-0-acetyl GD3 on the immunizing cells or the target cells for serological assays, rather than poor immunogenicity. We have recently shown that the immunogenicity of a series of gangliosides in the mouse as well as in man was inversely proportional to their expression on normal tissues. The low expression of 9-0-acetyl GD3 on normal tissues in man suggested it might be immunogenic. Since Cy + BCG-GM2 was more effective than the immunization approaches used previously, we were eager to apply it to induction of antibodies against GD2, GD3 and 9-0-acetyl GD3. We show here that 9-0-acetyl GD3 is highly immunogenic, as immunogenic as GM2, and that using this approach, GD2 is only slightly immunogenic and GD3 is not immunogenic at all.

9-0 acetyl GD3 was originally identified as a melanoma antigen by Cheresch et al. using the mouse monoclonal antibody D1.1 established by Stallcup (5, 17). These reports and a subsequent one by Thurin et al. have shown the distribution of 9-0-acetyl GD3 to be largely restricted to melanoma cells. No expression on normal tissues was detected. While in depth immunohistologic studies have not yet been reported, the distribution pattern on normal tissues of 9-0-acetyl GD3 is less than GD2 or GD3 and appears to be comparable to or more restricted than GM2. In addition, Tchuda et al. have reported that 9-0-acetyl GD3 is expressed on all melanoma cells, generally in amounts comparable to GM2 (2). These factors and our studies in the mouse suggesting immunogenicity would be inversely proportional to expression on normal tissues lead us to acetylate GD3, isolate fractions containing 9-0 acetyl GD3 and confirm the immunogenicity of these fractions in mice. The 9-0-acetyl GD3 fraction utilized here has been shown by NMR to contain GD3 acetylated on the 9 position of the second sialic acid and a second acetyl group on the lactose backbone. Compared to GD3, no additional acetyl groups or other differences were detected. This fraction is completely converted to GD3 by base treatment and appears identical to the 9-0-acetyl GD3 identified on melanoma cells by Thurin. It reacts with 9-0-acetyl GD3 monoclonal antibodies D1.1, ME311 and Jones. Immunologic similarity of this fraction to that on melanoma cells is confirmed by the reactivity with melanoma cells of anti-9-0 acetyl GD3 antibodies induced in our patients. A second highly immunogenic melanoma ganglioside has been identified.

We have reported previously that patients developing GM2 antibody after vaccination have delayed melanoma recurrence (12). We have recently initiated a randomized study involving larger numbers of patients to determine whether the Cy + BCG-GM2 vaccine contributes to this apparent improvement in prognosis, or whether induction of GM2 antibody is merely a prognostic indicator. Expression of individual gangliosides on different cells in the same biopsy as well as in different biopsies varies considerably. Antigenic heterogeneity and the prospect that antibodies against different gangliosides would be synergistic, provide strong motivation for development of a multivalent melanoma vaccine. We show here that antibody induced in melanoma

patients against GM2 and 9-0-acetyl GD3 are indeed synergistic, resulting in significant melanoma cell death in settings where neither alone were effective. Antibodies against GD3 would be expected to be especially potent, alone or in combination with these other antibodies, as GD3 expression on melanoma cells is generally 10-20-fold greater than that of GM2 or 9-0-acetyl GD3. Consequently, we have begun to explore approaches for increasing the immunogenicity of GD3.

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What is claimed is:

1. A composition for stimulating or enhancing in a subject to whom the composition is administered, production of antibodies against 9-O-acetyl GD3 ganglioside comprising an amount of a 9-O-acetyl GD3 ganglioside effective to stimulate or enhance antibody production in the subject and a pharmaceutically acceptable carrier.
2. A composition of claim 1 which additionally comprises an adjuvant.
3. A composition of claim 2, wherein the adjuvant is a microbial adjuvant.
4. A composition of claim 3, wherein the microbial adjuvant comprises *Salmonella minnesota* R595.
5. A composition of claim 3, wherein the microbial adjuvant comprises bacillus Calmette-Guerin.



6. A composition of claim 1, wherein the effective amount of 9-O-acetyl GD3 ganglioside comprises an amount between about 50 micrograms and about 300 micrograms.

7. A composition of claim 1, wherein the 9-O-acetyl GD3 ganglioside is purified from a biological source.

8. A composition of claim 7, wherein the biological source is a melanoma cell.

9. A composition of claim 7, wherein the biological source is milk.

10. A composition of claim 7, wherein the biological source is buttermilk.

11. A composition of claim 1, which additionally comprises purified GM2 ganglioside.

12. A method for stimulating or enhancing in a subject production of antibodies against 9-O-acetyl GD3 ganglioside comprising administering to the subject an effective dose of a composition of claim 1.

13. A method of claim 12, wherein the 9-O-acetyl GD3 ganglioside is bound to a microbial adjuvant.

14. A method of claim 12, wherein the 9-O-acetyl GD3 ganglioside is bound to the microbial adjuvant by a hydrophobic bond between the lipid portion of the 9-O-acetyl GD3 ganglioside and the cell membrane of the microbial adjuvant.

15. A method of claim 12, wherein the microbial adjuvant is *Salmonella minnesota* R595 or bacillus Calmette-Guerin.

16. A method of claim 12, wherein an effective amount of cyclophosphamide is administered to the subject prior to administering the composition.

17. A method of claim 16, wherein the cyclophosphamide is administered between about 3 days and about 7 days prior to the administering the composition.

18. A method of claim 16, wherein the effective amount of cyclophosphamide is between about 1 mg/m<sup>2</sup> and about 500 mg/m<sup>2</sup>.

19. A 9-O-acetyl GD3 ganglioside designated F2 characterized by the presence of a second acetyl group and the mass spectra shown in FIGS. 6A, 6B, and 6C.

20. A 9-O-acetyl GD3 ganglioside designated F3 characterized by the presence of 2 or more additional acetyl groups and recoverable from a mixture of acetylated derivatives of GD3 by a high pressure liquid chromatography.

21. A mixture of the 9-O-acetyl GD3 gangliosides F2 and F3.

22. A composition for stimulating or enhancing in a subject to whom the composition is administered, production of antibodies against 9-O-acetyl GD3 ganglioside comprising an amount of a 9-O-acetyl GD3 ganglioside of any of claims 38, 39 or 40 effective to stimulate or enhance antibody production in the subject and a pharmaceutically acceptable carrier.

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